Research Article

Title: Nanoscale characterization coupled to multi-parametric optimization of Hi5 cells transient gene expression

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Abstract

Polyethylenimine (PEI)-based transient gene expression (TGE) is nowadays a well-established methodology for rapid protein production in mammalian cells , but it has been used to a much lower extent in insect cell lines. A fast and robust TGE methodology for suspension Hi5 (*Trichoplusia ni*) cells is presented. Significant differences in size and morphology of DNA:PEI polyplexes were observed in the different incubation solutions tested. Moreover, minimal complexing time (< 1 min) between DNA and PEI in 150 mM NaCl solution provided the highest transfection efficiency. Nanoscopic characterization by means of cryo-EM revealed that DNA:PEI polyplexes up to 300 – 400 nm were the most efficient for transfection. TGE optimization was performed using eGFP as model protein by means of the combination of advanced statistical designs. A global optimal condition of 1.5 x 10^6 cell/mL, 2.1 µg/mL of DNA and 9.3 µg/mL PEI was achieved through weighted-based modelling of transfection, production and viability responses. Under these conditions, a 60 % transfection and 0.8 µg/10^6 transfected cell·day specific productivity was achieved. The TGE protocol developed for Hi5 cells provides a promising baculovirus-free and worthwhile approach to produce a wide variety of recombinant proteins in a short period of time.

Keywords: High Five cells, Polyethylenimine, Transient gene expression, Cryo-Electron microscopy, Dynamic Light Scattering, Design of Experiments

Introduction

The emergence of high-throughput techniques has brought under the spotlight the need for efficient and productive hosts to screen for a large number of protein candidates. CHO and HEK293 mammalian cell lines commonly serve to this purpose since they efficiently produce complex protein structures with adequate post-translational modifications (Walsh and Jefferis 2006). Likewise, insect cells have also proven to be useful systems to produce difficult-to-express proteins maintaining their biological activity (Fernandes et al. 2013). Furthermore, insect cells are capable to grow to high cell densities and they also have less strict culture requirements. Sf21 and Sf9 from *Spodoptera frugiperda*, Hi5 (High Five) and Tna38 from *Trichoplusia ni* are the most extensively used insect cell lines. In general terms, *Trichoplusia ni*-derived cell lines are the platforms selected for protein production since higher specific productivities are achieved compared to Sf9 and Sf21 cell lines. Typically, the baculovirus expression vector system (BEVS) is the preferred production methodology with regards to protein expression in insect cells. While the baculovirus-insect cell system has been successfully applied for the production of a number of target proteins, a long-time is still required from the cloning of the gene of interest (GOI) to obtain the baculovirus working stock (Roest et al. 2016). Besides, several limitations of this system have been encountered with virus-like particles at the downstream phase due to co-purification of the two specimens (Liu et al. 2013). A lack of consistency in the production of adeno-associated viruses (AAV) has also been reported and attributed to instability of the AAV cassette in baculoviruses during the expansion phase (Clément and Grieger 2016).

Growing interest has emerged on exploiting the benefits of insect cell lines for protein production devoid of BEVS methodology. Stable expression based on the transfection of a DNA plasmid encoding for the GOI with subsequent antibiotic based selection has been evaluated for different proteins with variable results (Shoja et al. 2015; Vidigal et al. 2018). However, stable gene expression is time-consuming when several GOI candidates have to be evaluated. In the same line, selecting a high producer clone from the stable expressing cell population entails a lot of effort which limits to a certain extent the applicability of this system for short-term applications (Fernandes et al. 2012).

Transient gene expression (TGE) is a methodology that enables rapid protein production in sufficient levels to be used in pre-clinical and early-clinical phases (Gutierrez-Granados et al. 2018). TGE has been extensively used in mammalian cells but less is known for its application to insect cell lines. First attempts were performed in serum-containing adherent cultures by means of liposome-derived reagents and low titers were obtained (Royl 1995; Lu et al. 1996). However, the use of these transfection carriers hindered the scaling-up of the process in terms of cost-efficiency and have been gradually relegated to stable gene expression. Alternatively, attention has been focused on using bulk chemicals such as polyethylenimine (PEI) to evaluate TGE in suspension cultures, with successful results in the field of mammalian cells.

In parallel, many efforts have been directed to developing serum-free fully chemically-defined media that support high insect cell densities but difficulties are encountered when replacing yeast hydrolysates. Recent studies have addressed PEI-based TGE of Hi5 cells but a lack of consistency due to the use of high hydrolysate containing media was reported (Shen et al. 2015; Mori et al. 2017). This lot-to-lot variability greatly hinders the standardization and characterization of the transient transfection process which may explain the few information available regarding the driving forces that mediate TGE in insect cells.

In this study, a robust and reproducible transient transfection method for suspension-adapted Hi5 cells using PEI is described. Characterization of the DNA:PEI complexes that mediate transient transfection was thoroughly evaluated by means cryo-electron microscopy (Cryo-EM) and dynamic light scattering techniques. Several incubation solutions and different times of DNA:PEI complex formation were also studied as factors potentially influencing the physical properties of transient transfection. Finally, advanced statistically response surface methodologies (RSM) coupled to weighted-based modelling of different responses were successfully applied towards defining an optimum transfection condition for Hi5 cells.

Materials and Methods

Cell line, media and culture conditions

The lepidopteran insect cell line used in this work is *Trichoplusia ni* BTI-TN-5B1-4 (High Five, cat. num. B85502, Thermo Fisher Scientific). Four different serum-free commercial media formulations for Hi5 cells were tested for the following parameters: cell growth, transfection and enhanced green fluorescent (eGFP) production. These include EX-CELL 405 from SAFC Biosciences (Saint Louis, MO, USA), Express Five SFM from Thermo Fisher Scientific (Waltham, MA, USA), Insect Xpress from Lonza (Verviers, Belgium) and Sf900III from Thermo Fisher Scientific (Grand Island, NY, USA). Cells were previously adapted to each medium prior to experimentation. Cell passaging was performed three times a week at a density of 2-4 x 10^5 cells/mL in 125-mL disposable polycarbonate Erlenmeyer flasks (Corning, Steuben, NY, USA) in 15 mL medium. All cultures were shaken at 130 rpm using an orbital shaker (Stuart, Stone, UK) and were maintained at 27°C.

Cell count and viability were measured with Nucleocounter NC-3000 (Chemometec, Allerod, Denmark) during 5-6 days. Maximum specific growth rates, μ_{max} (h^{-1}), and duplication times, $t_{1/2}$ (h) were determined from the data corresponding to the exponential growth phase.

Plasmid vectors

The plasmid vector used is the pIZTV5-eGFP (cat. num. V801001, Thermo Fisher Scientific) which harbours the immediate-early OPIE2 promoter from *Orgya pseudotsugata multicapsid nucleopolyhedrosis virus* (OPMNPV). The Sh *ble* resistance gene of this plasmid was fused in frame to a GFP in origin. The GFP portion of the Sh *ble* gene was removed by PCR cloning in order to eliminate fluorescence interference signals between GFP and eGFP. Briefly, pIZTV5-eGFP plasmid with the GFP gene at the Sh *ble* gene was PCR amplified using the following primer pair: GFPremove_fw: 5'-CGTAAGATCTCGCCATGGTTTAGTTCCTCAC-3' and GFPremove_rev: 5'-CGTAAGATCTATGGATGCCAAGTTGACCAG-3', then the PCR product was digested with *Bgl*II and ligated with T4 DNA ligase.

Standard transfection protocol

EGFP was produced by transient transfection of Hi5 cells with pIZTV5-eGFP plasmid DNA using 25 kDa linear polyethylenimine (PEI, PolySciences, Warrington, PA, USA). Linear PEI 25 kDa was prepared in ultrapure water at a concentration of 1 mg/mL with pH of 7 and was sterilized by filtration. The standard transfection protocol was defined according to preliminary experiments and was further optimized as detailed in the "Results" section. Prior to transfection, medium exchange was performed to exponentially growing cells by centrifugation at 300x*g* during 5 min. Cells were resuspended to 1 x 10^6 cell/mL in 15 mL medium. DNA and PEI complex formation was performed in 1mL incubation solution with DNA at 1 µg/10^6 added first and vortexed during 10 s; then PEI at 3 µg/10^6 was added to DNA and vortexed for 10 s. Dulbecco's Phosphate-Buffered Saline (DPBS) from Thermo Fisher Scientific (Logan, UT, USA) was selected as the DNA:PEI complexing solution for the medium screening phase but other solutions were also tested. These solutions include NaCl from Sigma-Aldrich (Saint Louis, MO, USA) and ultrapure water from Merck Millipore (Burlington, MA, USA). After 15 minutes of incubation at room temperature, the mixture was added to the culture. The percentage of eGFP expressing cells was assessed by flow cytometry using a BD FACS Canto II flow cytometer (BD Biosciences, San José, CA, USA) at different hours post transfection (hpt).

Fluorescence confocal Microscopy

The visualization of eGFP producer cells was performed using a TCS SP5 confocal microscope (Leica, Wetzlar, Germany). Transfected cells were dyed with 0.05% v/v of CellMask™ and 0.1% v/v of Hoechst from Thermo Fisher Scientific (Eugene, OR, USA) in order to stain the lipid membrane and cell nucleus respectively. A washing step was performed by centrifuging the cells at 300x*g* during 5 min and resuspending the pellet in fresh DPBS. Samples were placed in 35 mm glass bottom petri dishes with 14 mm microwell (MatTek Corporation, Ashland, MA, USA) for visualization.

Cryo Electron Microscopy

Morphology and qualitative particle size distribution of DNA:PEI complexes formed in different solutions was studied with a cryo transmission electron microscope (TEM). Briefly, 2 µL of sample

was blotted onto carbon copper or holey carbon 200 mesh copper (Quantifoil Micro Tools, Großlöbichau, Germany) grids previously subjected to a glow discharge treatment in a PELCO easiGlow™. Discharge Cleaning System (PELCO, Fresno, CA, USA). The samples were subsequently plunged into liquid ethane at −180 °C using a Leica EM GP workstation (Leica, Wetzlar, Germany) and observed in a JEM-2011 TEM operating at 200 kV (Jeol Ltd., Akishima, Tokyo, Japan). Samples were maintained at −180 °C during imaging and pictures were taken using a CCD 895 USC 4000 multiscan camera (Gatan, Pleasanton, CA, USA).

Determination of DNA:PEI complex size

DNA:PEI average complex size distribution was evaluated with a Zetasizer Nano ZS dynamic light scattering (DLS) instrument (Malvern instruments, Malvern, UK) with a He/Ne 633 nm laser at 173°. The hydrodynamic diameter and polydispersity index (PDI) were calculated with cumulative fit correlation at 25 °C and 0.8872 cP. DNA:PEI complexes were prepared as described previously and placed in disposable plastic cuvettes followed by automated experimental data collection with Zetasizer Nano software (Malvern instruments, Malvern, UK). Complex formation was observed up to 1 µm average DNA:PEI complex size which is the upper detection threshold of the DLS equipment. The kilocounts per second (kcps) were also monitored and compared between samples according to the standardized values given by the Zetasizer Nano software.

eGFP quantitation

EGFP producing cells were harvested by centrifugation at 3000 *g* for 5 min. Pelleted cells were then subjected to a three freeze-thaw cycles (2h freezed at -20ºC and thawed at 37ºC for 0.5 h). Samples were vortexed for 5 seconds between cycles. Lysed pellets were resuspended in 0.5 mL of TMS buffer (50 mM Tris-HCl, 150 mM NaCl, 2 mM $MgCl_2$, pH 8.0) and centrifuged at 13700 x *g* during 20 min. EGFP green fluorescence was measured with a Cary Eclipse fluorescence spectrophotometer (Agilent Technologies, Santa Clara, CA, USA) at room temperature set as follows: λ_{ex} = 488 nm (5 nm slit), λ_{em} = 500 - 530 nm (10 nm slit). Relative fluorescence unit values (R.F.U) were calculated by substracting fluorescence unit values of negative non-transfected cultures. EGFP mass concentration

was determined using a standard curve by a linear correlation of a known concentration of eGFP (BioVision, Milpitas, CA, USA) and the associated fluorescence intensity in R.F.U:

$$\text{eGFP (mg/L)} = (\text{R. F. U} - 1.0122)/32.564 \quad (1)$$

where eGFP is the estimated concentration of eGFP protein and R.F.U is the measured eGFP fluorescence intensity in the samples. Sf900III medium and 0.1 mg/mL quinine sulphate solution were used as control patterns to normalize R.F.U values between different experiments.

Optimization of transient transfection using DoE

Transient transfection was optimized to maximize eGFP specific production using Design of experiments methodology (DoE). In addition, the percentage of eGFP positive cells and cell culture viability were also measured and considered as responses towards completely defining the transient transfection process with the final aim of finding a global process optimum. Viable cell concentration at the time of transfection, DNA and PEI concentration were the independent variables selected for optimization.

Box-Behnken design

A Box-Behnken design was used in order to define the optimal condition for each response and the independent variables to be studied. The three variables were screened at three levels coded as follows: low level, -1; medium level, 0; high level, +1; as indicated in Table 1. The obtained results were fitted to a second-order polynomial equation by non-linear regression analysis for each response (Eq. 2):

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i \cdot X_i + \sum_{i=1}^{k} \beta_{ii} \cdot X_i^2 + \sum_{i=1}^{k} \sum_{j>1}^{k} \beta_{ij} \cdot X_i \cdot X_j + \varepsilon \quad (2)$$

where Y is the response variable (eGFP specific production in R.F.U/10^6 transfected cells, percentage of eGFP positive cells or cell culture viability in percentage), β_0 is the model intercept term, β_i the linear coefficient, β_{ii} the quadratic coefficient, β_{ij} the interaction coefficient, X_i and X_j are the studied variables (Viable cell concentration at the time of transfection, DNA and PEI concentration) and ε is the experimental error. The different model equations based on model Eq. 2 were obtained using the R

Software (R Development Core Team, Vienna, Austria) by means of the *Frf2*, *car* and *RcmdrPlugin DoE* packages (Kuhnt and Rudak 2013). These equations were used to predict the optimal values of the independent variables using the L-BFGS-B quasi-Newton algorithm implemented in the *optimx* package of the R software. Three-dimensional plots were generated with the *gplots* package to facilitate model interpretation and were readapted with Adobe Illustrator CS6 (Adobe Systems Incorporated, San José, CA, USA).

Multiple response optimization using desirability functions

The best overall condition considering the three model equations was determined using the *desirability* package from the R software. However, several modifications in the basic code of this package were implemented in order to successfully apply this methodology. The design space was fixed as circular because of the Box-Behnken design used for the optimization and values outside this space were not considered. Different ranges were selected for each response according to the experimental data and a relevance value (*s* value) was also chosen depending on the importance of the model equation (Eq. 3):

$$d_n = \begin{cases} 0 & if\ Y_n < LL \\ \left(\frac{Y_n - LL}{UL - LL}\right)^s & if\ LL \leq Y_n \leq UL \\ 1 & if\ Y_n > UL \end{cases} \qquad (3)$$

where d_n is the desirability response function for each model equation in [0 – 1] scale, *s* accounts for the relevance value given to the equation, Y_n is the model equation (in this case n = 3), *LL* and *UL* are de lower and upper limits of each equation, respectively. Greater *s* values make the desirability value d_n more difficult to be satisfied and are associated to the most important model equations. The different *N* desirability functions are then combined to achieve an Overall Desirability function *OD* (Eq. 4):

$$OD = dmax\left(\prod_1^N d_n\right)^{1/N} \qquad (4)$$

where *OD* is the overall desirability to be maximized, d_n is the desirability response function for each model equation in [0 – 1] scale and *N* is the number of desirability functions. The *OD* value is the output of applying the geometric mean to the *N* desirability functions which has the property that if

any model equation is undesirable (d_n = 0), the overall desirability is also not satisfied (*OD* = 0). After defining the different items that integrate the *desirability* code, an iteration process seeks for the best *OD* ending when the best condition maximizing the *OD* is found.

Statistical analyses

Statistical analyses of the different models were performed using R Software by means of the *car*, *MASS* and *faraway* packages. The quality of the regression of the model equations was evaluated by the coefficients R^2 and R^2_{adj}. The overall significance of the model was determined by analysis of variance (ANOVA) *F*-test whereas the significance of each coefficient was determined by the corresponding *t*-test. The lack-of-fit (LOF) test was used to evaluate differences between experimental and pure error of the models. Normality of the residuals was evaluated by means of the Shapiro-Wilk test whereas constant variance of the residuals was assessed by means of the Breusch-Pagan test.

Results

Media screening for cell growth and transfection efficiency

Four different serum-free commercially available media were selected for Hi5 cell culture in suspension (Osz-Papai et al. 2015; Palomares et al. 2015). All formulations tested (EX-CELL 405, Express Five SFM, Insect Xpress and Sf900III) are serum-free. The first three media contain hydrolysates to a certain extent whereas Sf900III is the only medium with low-hydrolysate content and animal origin-free compounds (Chan and Reid 2016). Nonetheless, the exact composition of the media is proprietary of the manufacturers. In preliminary experiments, cell growth of Hi5 cells in the different formulations was evaluated in batch culture (Fig. 1A). Unexpectedly, cells did not adapt to grow in Express Five SFM and therefore this medium was not included in further experiments. For all other media, cells maintained a high viability (> 90%) until 96h coinciding with maximum cell density. Insect Xpress medium supported the highest maximum cell concentration (5.0 ± 0.1 x 10^6 cells/mL) with a doubling time of 15.5 ± 0.4 h. Hi5 cells grew up to 4.5 ± 0.3 x 10^6 cells/mL with a doubling time of 20.5 ± 0.5 h in Sf900III medium whereas 3.6 ± 0.3 x 10^6 cells/mL and a doubling time of 17.8 ± 1.8 h was achieved in EX-CELL 405 medium.

Intracellular eGFP production upon transfection with the standard protocol was also studied through sampling every 24 h during 5 days. Transfection was performed with DNA and PEI pre-complexation in DPBS for 15 min. The percentage of eGFP positive cells peaked at 48 hpt in all media formulations, being highest in Sf900III (~35 %) while ~21 and ~23 % transfection was achieved in Insect Xpress and EX-CELL 405, respectively (Fig. 1C). The percentage of eGFP positive cells was maintained in Sf900III up to 96 hpt whereas it decreased in the other two media beyond 48 hpt.

Green fluorescent cells were visualized under confocal fluorescence microscoe at 48 hpt. Green fluorescence due to eGFP expression was observed in the cytoplasm of transfected cells (Fig. 1B).

Then, the kinetics of eGFP expression upon transient transfection was evaluated by monitoring intra and extracellular fluorescence intensity at different times post-transfection. Maximum eGFP production was obtained in all media at 72 hpt, being the highest in Sf900III (~150 R.F.U) as shown in Fig. 1D. In contrast, lower production levels were reached both in Insect Xpress (~100 R.F.U) and EX-CELL 405 with ~60 R.F.U. Barely no eGFP expression was detected in the supernatant of the different media formulations until 96 hpt (Fig. 1E) and only low amounts were detected afterwards coinciding with the drop in cell viability. In this sense, protein leakage from death cells could explain the presence of eGFP in the supernatant which is supported by the decrease in intracellular eGFP from 96 hpt on. Distinct agitation conditions during transfection (80, 130, 150, 170 rpm) were also evaluated with no significant differences neither in the transfection percentage nor in eGFP production (data not shown).

Sf900III was the medium selected for further optimization experiments since it showed the best transfection and production levels and also good cell growth. Of note, this medium is devoid of animal-derived compounds and also has a low-hydrolysate content. These features contribute to diminish experimental variation due to lot-to-lot variability and makes it an interesting platform towards defining a robust bioprocess.

Size of Hi5 cells adapted to grow in Sf900III was measured under a confocal microscope resulting in a diameter of 16.3 ± 2.2 µm (50 cells were measured). This value was in good agreement with the

results obtained by (Sander and Harrysson 2007) for Hi5 cells adapted to Express Five medium (16.3 µm). Therefore, Hi5 cell size does not probably change in a significant manner with regards to the culture medium used.

Selection of the DNA:PEI complexing solution

Different solutions were investigated for Hi5 cells transient transfection according to their use as DNA:PEI complexing media (van Gaal et al. 2011; Raup et al. 2017). These incubation solutions include 3 and 150 mM NaCl, DPBS, ultrapure water and the cell culture medium Sf900III. Cell transfection with previous DNA:PEI complexation in the different incubation solutions was performed according to the standard transfection protocol. Direct transfection without prior pre-complexation was also evaluated since it has been reported to be efficient in a variety of cell lines (Hacker et al. 2013; Gutierrez-Granados et al. 2016). To this purpose, sequential addition of PEI followed by DNA was carried out since the opposite combination has shown little efficiency on transfection as described elsewhere (Shen et al. 2013). Flow cytometry analysis showed comparable transfection efficiencies around 30 - 35% in 3 and 150 mM NaCl solutions and DPBS at 48 hpt, being the highest in 150 mM NaCl (Fig. 2A). Lower transfection efficiencies of ~20 - 25% were obtained with DNA:PEI complexes pre-formed in water and Sf900III medium whereas addition of PEI and DNA without prior pre-complexation resulted in the worst overall condition (~10%).

Assessment of eGFP expression at 72 hpt revealed that maximum eGFP production was obtained in the incubation solutions with the highest salt content (Fig. 2B). Conversely, smaller production levels where achieved both in 3 mM NaCl and ultrapure water while no pre-complexation of DNA and PEI showed the lowest production. Interestingly, there was no correlation between transient transfection efficiency and eGFP production. According to these results, 150 mM NaCl was selected as the DNA and PEI incubation solution since it showed the best transfection yield and a good production level of eGFP.

Characterization of DNA:PEI complexing process

DNA:PEI complexes pre-formed in the different incubation solutions were examined with DLS to better characterizing the nature of the species that mediated transfection and production in Hi5 cells. PEI and pIZTV5-eGFP solutions were mixed according to the standard transfection protocol in different complexing solutions: Sf900III medium, 3 and 150 mM NaCl, DPBS and ultrapure water. The whole population of complexes incubated in Sf900III, 150 mM NaCl and DPBS experienced a dynamic shift of increasing in size during the time period tested (Fig. 3A). Interestingly, similar aggregation patterns were observed between 150 mM NaCl and DPBS. Polyplexes incubated in 150 mM NaCl exhibited a higher polydispersity and grew up to 1 µm within 25 min whereas in DPBS this complex size was attained in 35 min. Polyplexes seemed to stabilize at size of approximately 1 µm since higher structures could not be efficiently tracked due to DLS equipment limitations. DNA:PEI complexes incubated in Sf900III medium revealed the coexistence of two populations where the smaller one decreased over time whilst the higher population increased (Figure S1). This would be mainly attributed to the aggregation of smaller polyplexes that triggered the formation of higher structures. On the contrary, a very slow aggregation tendency was observed in ultrapure water and 3 mM NaCl. In both solutions, two different populations were encountered but no clear aggregation tendencies were observed in any of them over the studied time period. A smaller DNA:PEI complex population was clearly more abundant in these two solutions, stabilizing at 50-60 nm in 3 mM NaCl and at 60-70 nm in ultrapure water. The kilocounts per second (kcps) decreased over time in all the incubation solutions, especially in those with the highest salt content (Fig. 3B). Ultrapure water and 3mM NaCl solutions showed a faint decline in the kcps over time which was in agreement with DNA:PEI complex size stabilization previously described. This phenomenon could be in turn related either with an aggregation or precipitation process of DNA:PEI complexes but the latter was discarded since no sample precipitation was observed. Besides, 150 mM NaCl, Sf900III medium and to a lesser extent DPBS were the complexing solutions with highest kcps during the time range tested. Higher kcps values could be a consequence of an increase in either the number or size of DNA:PEI complexes

or both. It could be then argued that bigger structures were present in 150 mM NaCl, Sf900III medium and DPBS but not ascertainment of also higher concentration of complexes could be made.

Addition of DNA to the different incubation solutions did not trigger any aggregation process, but 100-200 nm particle formation was observed in Sf900III (Figure S2). PEI addition triggered an aggregation process only in Sf900III medium which would indicate that PEI would be interacting with negatively charged medium compounds other than DNA. (Figure S3).

Morphology of DNA:PEI complexes in the different incubation solutions was also studied by means of Cryo-EM. Organisation of DNA and PEI into particles was observed in all the incubation solutions (Fig. 4). These complexes appeared as black nearly round electron-dense structures which could be clearly identified in Sf900III medium, 150 mM NaCl and DPBS. In these solutions, DNA:PEI polyplexes seem to be formed by the aggregation of smaller pieces of complexes of around 200-400 nm and grew up to > 1 µm structures. Otherwise, DNA:PEI complexes smaller than 100 nm were observed both in ultrapure water and 3 mM NaCl (Fig. 4C-D). In these two solutions, small complexes were accompanied by bigger less dense stains that could be related to unbound DNA as compared to DNA control samples (Figure S4). Moreover, PEI addition to each incubation solution did not unleash particle formation except for Sf900III medium which is in agreement with the results obtained with DLS (Figure S5).

DNA and PEI complex formation kinetics uncovered that incubation time between the two species could influence the transfection of Hi5 cells. To value this, a transfection experiment with DNA:PEI complexes pre-formed in 150 mM NaCl during different incubation times (< 1, 5, 10 and 15 min) was performed. A staggered increase in transfection was achieved as the incubation time between DNA and PEI decreased which corresponded to small DNA:PEI polyplexes (Fig. 3C). Smaller DNA:PEI complexes up to 300 – 400 nm were observed during < 1 min incubation comapred to DNA:PEI complexes incubated during 15 min (Fig. 4E-F). In this sense, the synergy obtained by applying dynamic light scattering and cryo-electron microscopy techniques revealed that DNA:PEI polyplexes

minimally incubated in 150 mM NaCl were the most efficient upon transfection and production in Hi5 cells.

Optimization of transient eGFP production by DoE

Different variables affecting the transfection of Hi5 cells were evaluated in the previous sections in order to diminish the number of parameters to be evaluated by means of more elaborated statistical techniques. DNA, PEI and cell concentration at the time of transfection were selected as the most influencing variables on eGFP transfection and production as previously reported for other cell lines (Thompson et al. 2012; Fuenmayor et al. 2018). To this purpose, a DoE-based approach was used to achieve an optimum condition with a reduced number of experiments in a reasonable time. Additionally, variability caused by nuisance factors within the same experiment and between different experiments was carefully considered. As an illustration of this phenomenon, it was observed that a high cell passage number of >30 after thawing entailed a decrease of about 10-20% in transfection of Hi5 cells (data not shown) which could interfere with the interpretation of the relevant variables. As a consequence, the cell age window was carefully monitored in all experiments, assuring that cells were not cultured beyond 20 passages. Similar performances were also reported in (Bollin et al. 2011). Of note, different Sf900III medium lots and distinct PEI 25 kDa stocks were used along the experimentation phase without relevant variation in the outcomes.

Response surface methodology and experimental space boundaries

A three-factor, three-level Box-Behnken design was selected to optimize DNA, PEI and cell concentration at transfection as it is an efficient methodology regarding the number of experimental runs to be performed and the statistically relevant information that can be obtained (Montgomery 2012). The range for cell concentration was chosen according to the cell growth exponential phase of Hi5 cells in Sf900III medium whereas DNA concentration was selected based on literature (Backliwal et al. 2008; Cervera et al. 2013). In the case of PEI, concentration ranges were picked based on empirical toxicity assays (Figure S6). Since PEI had a toxicity effect on cells, the upper limit was set

at 11 µg/mL and the lower at 5 µg/mL. Working ranges for each variable at different levels -1, 0 and +1 are presented in Table 1.

Box-Behnken design matrix consisted in 15 experiments in which the central point was triplicated to account for the pure experimental error. Cells seeded at 0.3 x 10^6 cell/mL were amplified during 48 h and then subjected to a medium exchange to fresh Sf900III medium prior to transfection with 1 mL of DNA:PEI complexes minimally incubated (< 1 min) in 150 mM NaCl. Specific production (R.F.U/10^6 cell), transient transfection (% of eGFP positive cells) and viability (% of viable cells) were evaluated as responses in this study (Table 1). The data were fitted to a second-order polynomial model by the least squares method and a different model was obtained for each one of the response variables based on Eq. 2. The statistical significance of each model equation was confirmed by ANOVA analysis as depicted in Table 1. Model equation were subjected to a refinement process to eliminate non-statistically significant terms (NS) based on the hierarchy principle (Peixoto 1987).

Three-dimensional plots were built according to model equations in order to detect synergies between DNA, PEI and viable cell concentration (Fig. 5A-I). Noticeably, the three independent variables had a strong influence on specific production being viable cell concentration at transfection the most relevant one (Table 1). In all cases, DNA, PEI and cell concentrations close to the space boundaries of the design revealed a decreased outcome in terms of eGFP specific production. Hi5 cells transfected at mid concentration (level = 0) showed the highest production rate. In this sense, the optimum condition maximizing specific production for the three variables was located in the central region of the design space (Fig. 5A-C) which corresponded to 2.3 µg/mL of DNA, 8.1 µg/mL of PEI and 2.1 x 10^6 cell/mL at the time of transfection.

A different behavior was observed regarding transient transfection efficiency, being viable cell concentration the most significant variable and to a lesser extend DNA and PEI concentrations (Fig. 5D-F). In fact, the lower the cell concentration the higher the transfection efficiency, which was more pronounced with low amounts of DNA and high concentrations of PEI (Fig 5D). A statistically significant interaction between cell and DNA concentration was identified (p-value = 0.013, see Table

1) and indications of a possible interaction between DNA and PEI were also encountered. Also, high DNA concentrations did not trigger efficient Hi5 cell transfection with any PEI combination tested probably due to an imbalance in DNA:PEI complex formation (Fig. 5F). Then, the optimum condition maximizing transient transfection was found as 1 µg/mL of DNA, 11 µg/mL of PEI and 1 x 10^6 cell/mL at the time of transfection.

The effect of DNA, PEI and cell concentration on culture viability was also monitored to better understand cell state at the time of harvest. Viable cell concentration at the time of transfection was the most influencing variable whereas PEI and DNA concentration had little and no effect, respectively. Cells transfected at a higher concentration showed an earlier decline in cell viability when compared to Hi5 cells transfected at a low concentration (Fig. 5G-I). Interestingly, high PEI concentrations within the design range exhibited a slight beneficial effect on viability of high concentrated cells. This could be attributed to better transfection efficiencies obtained at high PEI concentrations with a reduced growth rate of transfected cells compared to non-transfected cells. On the contrary, DNA concentration did not impact on cell viability as observed in Fig. 5G-I where DNA concentration increases from 1 to 3 µg/mL and viability remained unchanged. In this case, the optimum for DNA, PEI and cell concentration was 1 µg/mL of DNA, 5 µg/mL of PEI and 1 x 10^6 cell/mL.

Multiple response optimization with desirability functions

The assessment of Hi5 cells transient transfection by means of monitoring several responses yielded different optimal conditions in each of the model equations. The purpose was to obtain an overall transfection condition integrating the different responses in order to avoid situations involving high transfection efficiencies but low productivities or vice versa. To do this, a weighted-based modelling approach of the three responses consisting in the application of desirability functions was implemented. Weight assignation to the responses (s – value) was performed according to the priority given to each of the modelled equations. In this regard, preference was assigned to the percentage of transfection (s = 2) and specific production (s = 1.5) whilst cell viability at harvest time was considered as easily to be accomplished (s = 1). The transformed d_n responses were then combined in

a unique overall desirability (*OD*) function and the best condition maximizing *OD* was found by applying Eq. 4. The combination of the three responses resulted in 2.1 µg/mL of DNA, 9.3 µg/mL of PEI and 1.5 x 10^6 cell/mL as the global optimum condition with an *OD* score of 0.47. A sensitivity analysis was also performed by changing the *s* values of the different equations resulting in very similar optimum conditions that confirmed the robustness of the calculation (data not shown).

Validation of the optimized model

A confirmation experiment was carried out to corroborate the optimal condition predicted by the combination of RSM and desirability functions (Fig. 6). Under these conditions, the experimental output was a maximum eGFP production of 5.5 ± 0.5 mg/L and 58.1 ± 0.9 % transfection which was within the calculated prediction interval of the overall optimum (Table 2). Cell culture viability was 80.6 ± 0.6 % at the time of maximum production (72 hpt) in good agreement with model prediction (Fig. 6A). EGFP specific productivity was calculated as 0.8 µg/10^6 transfected cell·day in the optimal condition.

Posar aquí un subtitol d'aquesta part de comparació (Comparison between Hi5 and HEK 293 transient gene expression)

Finally, the optimal transfection condition was compared to the optimum obtained in a previous work with mammalian HEK293SF cells (Cervera et al. 2013), a stablished cell line for protein production by means of TGE (Backliwal et al. 2008; Bollin et al. 2011). Maximum Hi5 viable cell concentration and transfection (~60%) was achieved at 48 hpt whereas HEK293SF cells did between 72-96 hpt (~70%). The same trend was observed for maximum eGFP production, in this case 72 and 96 hpt for Hi5 and HEK293SF cell lines, respectively (Fig. 6C). Interestingly, 1.3-fold more eGFP was produced in Hi5 cells and the comparison considering the optimal harvest time for each cell line yielded a 2.3-fold increase in specific productivity of Hi5 over HEK293SF cells (0.4 µg/10^6 transfected cell·day).

Discussion

The development of production strategies in insect cells devoid of baculovirus is gaining increasing interest. Transient gene expression has been successfully used for the expression of a variety of

proteins in different mammalian cell lines (Gutierrez-Granados et al. 2018). Among the available transfection methodologies, liposome-based reagents or electroporation methods do not guarantee the scalability or cost efficiency of the process and have been gradually relegated to the generation of stable cell lines (Vidigal et al. 2018). In this regard, affordable transfection carriers such as polyethylenimine have become the gold standard for transient transfection purposes.

Recently, some publications have addressed PEI-based transient transfection in Hi5 cells reaching ~150 mg/L of TNFR:Fc (Shen et al. 2015) and ~120 mg/L Fab fragment (Mori et al. 2017) with specific productivities ranging from 7.1 to 8.7 µg/10^6 transfected cell·day, respectively. Difficulties were found to compare existing transfection methodologies since they considerably depends on the target recombinant protein, plasmid promoter, codon-optimization, enhancer regions and post-regulatory elements (Lu et al. 1997; Román et al. 2016). For example, a 10-fold difference in specific productivity is observed for the production of TNFR:Fc (3.5 µg/10^6 transfected cell·day) and eGFP (0.3 µg/10^6 transfected cell·day) in the same host (Shen et al. 2013; Bleckmann et al. 2016). In this regard, differences in specific productivity of the system here presented with the aforementioned publications could be attributed to the expression of different proteins. Interestingly, a 2-fold increase in specific productivity was achieved in the optimized system developed in comparison to the most recent published transient eGFP production in Sf9 insect cells (Radner et al. 2012; Bleckmann et al. 2016).

Existing PEI-based TGE methods of Hi5 cells use animal-compound and high-hydrolysate content culture media that could entail several limitations in terms of reproducibility and standardization in the production of biologicals (Shen et al. 2015). Thereof, the properties of DNA:PEI complexes could be subjected to lot-to-lot variations in the culture media which would compromise the applicability of this system. This variability hinders the appropriate characterization of TGE in insect cells and consequently many variables influencing transfection remain undefined. Here, the use of an animal origin-free and low-hydrolysate containing medium favoured to better understand the synergies

between several variables affecting TGE and should also contribute to develop more reproducible bioprocessing methodologies.

Dynamic light scattering and cryo-electron microscopy were used to determine several properties of DNA and PEI complexing process. In general lines, DLS provided information about the mean size of DNA:PEI complexes and the co-existence of more than one population. Polyplexes pre-formed in Sf900III, 150 mM NaCl and DPBS showed a mean size population increase with longer incubation times between DNA and PEI. On the contrary, DNA and PEI incubation in ultrapure water and 3 mM NaCl did not trigger any aggregation process during time period tested. The latter confirmed that the presence of salt in the incubation solutions enhanced the aggregation of DNA and PEI (Sang et al. 2015). Furthermore, smaller polyplexes present in the incubation solutions with lower salt content (ultrapure water and 3 mM NaCl) displayed similar transfection efficiencies to those pre-formed in the solutions with higher salt content (Sf900III, 150 mM NaCl and DPBS). This unveiled that small DNA:PEI polyplexes were the ones governing efficient transfection of Hi5 cells, similarly to evidences found in other cell types (Raup et al. 2017).

Interestingly, there was no correlation between transient transfection efficiency and eGFP production. For instance, DNA:PEI complex formation in 3 mM NaCl showed a similar transfection efficiency compared to 150 mM NaCl but approximately only one third of the production in 150 mM NaCl was achieved in the former. The opposite occurred with DNA:PEI complex formation in Sf900III, where a smaller transfection efficiency compared to 3 mM NaCl was obtained but more than 2-fold eGFP production was reached. This could be attributed to a better condensing capability of DNA in complexes pre-formed in higher salt-containing solutions compared to the small complexes obtained in low salt conditions. The former could probably load more DNA and boost the production of eGFP which could be evidenced with higher fluorescence intensities of transfected cells. In this sense, salt content in the solution of DNA and PEI polyplex formation was revealed as an important variable strongly influencing protein expression.

Cryo-EM helped to gain insight into the morphology of these particles considering recent studies highlighting the relevance of this property in DNA and drug delivery approaches (Chu et al. 2015; Chen et al. 2016). The advantage of using Cryo-EM was the visualization of different structure typologies and particle assemblies in aqueous solution that is generally the natural occurring process. In fact, other systems like Scanning or Transmission Electron Microscopy involve a sample pre-treatment with either drying or staining that may complicate the resolution of the specimen under evaluation. Cryo-EM revealed the presence of polyplexes higher than 1 µm which could not be efficiently detected by DLS and also confirmed the existence of diverse DNA:PEI complex populations in the different incubation solutions tested. These results differ from previous reported observations (Glaeser 2016) where DNA:PEI complexes structures higher than 1 µm could not be described in detail.

Optimization of viable cell concentration at time of transfection, DNA and PEI concentrations was performed by means of a DoE-based approach, a methodology that enables to achieve an optimum condition with a reduced number of experiments in a reasonable time (Montgomery 2012; Gutierrez-Granados et al. 2016). These three variables were used to monitor and model the three responses under consideration obtaining different optimal conditions. In fact, low cell concentrations improved the transfection yield whereas specific protein production increased at high cell concentrations. Strategies involving graphical optimization and the application of desirability functions were firstly considered towards the obtaining of a global optimal condition. Graphical optimization is a tedious methodology when more than two responses are to be optimized (Bezerra et al. 2008), consequently the mathematical-based approach on desirability functions was finally selected (Derringer and Suich 1980). This technique consisted in transforming each model equation to a dimensionless desirability scale d_n ranging from zero (undesirable response) to one (optimal response) as depicted in Eq. 3. Besides, each equation was given a weight (s value) according to the importance of each response. Low s values made the equation to more easily satisfy desirability maximization whereas large s values narrowed the possible responses satisfying this criterion. The transformed d_n responses were

then combined in a unique overall desirability (*OD*) function and the best condition maximizing *OD* was found by applying Eq. 4. Of note, the target of this optimization procedure was to find the best condition but not to get an *OD* = 1 since the latter is completely dependent on the shape of the responses and space boundaries (Vera Candioti et al. 2014). Similar approaches combining the use of DoE and desirability functions were found (Paillet et al. 2011; Islam et al. 2015) but here we demonstrate that this methodology is also applicable in a license-free software environment.

In conclusion, a robust and reproducible baculovirus-free transient gene expression methodology of Hi5 cells is presented. Characterization of DNA:PEI complexes by means of Cryo-EM and DLS techniques revealed that up to 300-400 nm size polyplexes were the most efficient in transfecting Hi5 cell line. Also, DNA:PEI complex formation in 150 mM NaCl under minimal complexing time (< 1 min) was proven as the best transfection and production condition. An optimal transfection condition was achieved by means of combining RSM and desirability functions which represents the first study dealing with multi-parametric optimization in TGE. The developed Hi5 cell line TGE protocol provides an alternative to mammalian cell lines to rapidly screen a wide variety of recombinant proteins within 3 days of transfection. Future efforts towards scale-up of this process should allow the development of a rapid, efficient and flexible protein production platform for therapeutic, diagnostic or structural biology purposes.

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Compliance with Ethical Standards

Conflict of interest

The authors declare that they have no competing interests.

Ethical approval

This article does not contain any studies with human participants performed by any of the authors.

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Figure legends

Figure 1. Growth and transfection kinetics of High Five cells in batch culture in different media. (A) Exponentially growing cells were seeded at 0.3 x 10^6 cells/mL in 125-mL flasks. Cell density and viability were determined every 24 h in each culture media. Mean values ± standard deviation of triplicate experiments are represented. (B) Confocal fluorescence microscopy image of Hi5 producer cells at 48 hpt. Cell nucleus was stained with Hoechst (blue) and membrane was stained with CellMask™ (red). Green fluorescence refers to the production of intracellular eGFP molecules. (C-E) Transfection and production of intracellular eGFP in different culture media. Exponentially growing cells were transfected at 1.0 x 10^6 cells/mL in 125-mL flasks. Hi5 cells transfection efficiency (C), intracellular eGFP production in pellet (D) and supernatant in relative fluorescence units (E) were measured. Mean values ± standard deviation of triplicate experiments are represented.

Figure 2. Transfection and eGFP production kinetics of High Five cells in batch culture in Sf900III medium with DNA:PEI complexes pre-formed in NaCl 3 and 150 mM, DPBS, Sf900III, ultrapure water and direct addition of PEI + DNA. (A) Hi5 cells transfection efficiency. Exponentially growing cells were transfected at 1.0 x 10^6 cells/mL in 125-mL flasks. (B) EGFP production in pellet in relative fluorescence units. A negative control consisting in DNA addition was also included. Mean values ± standard deviation of triplicate experiments are represented.

Figure 3. DNA:PEI complex kinetics in different solutions. (A-B) DLS measurement of mean DNA:PEI complex size (nm) and kilocounts per second (kcps) in NaCl 3 and 150 mM, ultrapure water, DPBS and Sf900III medium. (C) Hi5 cells transfection efficiency upon transfection with DNA:PEI complexes pre-formed in NaCl 150 mM during various incubation times. Mean values ± standard deviation of triplicate experiments are represented.

Figure 4. Cryo-electron microscopy images of DNA:PEI complexes in different incubation solutions. (A-E) DNA:PEI complexes incubated during 15 min in Sf900III, DPBS, ultrapure water, NaCl 3 mM and NaCl 150 mM, respectively. (F) DNA:PEI complexes incubated in NaCl 150 mM and subsequently added to cell culture (< 1 min).

Figure 5. Response surface graphs based on Box-Behnken experimental results. (A-C) Specific production of cell culture lysates as a function of DNA, PEI and viable cell concentration at 72 hpt. (D-F) Percentage of eGFP positive cells as a function of DNA, PEI and viable cell concentration at 48 hpt. (G-I) Cell culture viabilities as a function of DNA, PEI and viable cell concentration of DNA, PEI and viable cell concentration at 72 hpt. The three dimensional graphs were constructed by depicting two variables at a time and maintaining the third one at a fixed level.

Figure 6. Model validation and comparison to HEK293SF cell line. (A) Cell growth of Hi5 and HEK293SF cell lines transfected at their optima, respectively. (B) C Percentage of eGFP positive cells. (C) Intracellular eGFP concentration in cell culture pellets. Cell density, viability, % eGFP positive cells and eGFP production were measured daily. Mean values ± standard deviation of triplicate experiments are represented.